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ORIGINAL ARTICLE

Effect of Chitosan Oligosaccharides on the Growth of *Bifidobacterium* Species

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Abstract

The aim of this study was to determine the effect of chitosan and chitosan oligosaccharides (COS), prepared by enzymatic hydrolysis with cellulase from *Trichoderma reesei*, Celluclast® on the growth of *Bifidobacterium* sp. The growth of the two bacteria strains were determined every 12 h for 48 h under anaerobic incubation at 37 °C in four MRS media containing lactose, COS, chitosan and inulin. The bacteria cell growth in substrate-added medium increased significantly after 48 h of incubation, except for the chitosan medium. COS was found to have a similar growth effect on *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 when compared with inulin and lactose. The pH of medium containing COS, inulin and lactose fermented with *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 decreased rapidly after 12 h. *B. bifidum* ATCC 11863 showed the highest specific growth rate at 12 h. The results revealed that COS support the growth of probiotic bacteria, thus indicating that COS has the potential as new prebiotic source in the functional food industry.

Keywords: Chitosan; cellulase; enzymatic hydrolysis; chitosan oligosaccharides; *Bifidobacterium* species.

Introduction

The carbohydrates can be classified according to their molecular size or degree of polymerisation (DP) into monosaccharides, oligosaccharides or polysaccharides. IUB-IUPAC nomenclature defines oligosaccharides as saccharides containing 3 to 10 sugar moieties. In terms of physiological properties, carbohydrates can be classified into digestible and non-digestible. The non-digestible oligosaccharides (NDOs) are low molecular weight carbohydrates of intermediates in nature between simple sugars and polysaccharides. They can be obtained by direct extraction from natural sources, or produced by chemical processes hydrolysing polysaccharides, or by enzymatic and chemical synthesis from disaccharides. The NDOs are known to possess important physicochemical and physiological properties that made them as potential candidate as prebiotics (Gibson et al., 2004; Mussato and Mancilha, 2007; Roberfroid, 2007; Licht et al., 2011). Enrichment of diet

with NDOs is believed to improve the gut microecology such as bacterial populations, biochemical profiles and physiological effects (Crittenden and Payne, 1996; Voragen, 1998; Ziemer and Gibson, 1998; Roberfroid and Slavin, 2000).

Over the past decade, many food products with prebiotic compounds and probiotic microorganisms have been produced (Holzapfel and Schillinger, 2002; Tuohy et al., 2003). International Scientific Association for Probiotics and Prebiotics (ISAPP) defines prebiotics as selectively fermented, dietary ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health. A prebiotic is not hydrolysed in the small intestine and that it is selectively fermented in the colon by certain beneficial members of the colonic microbiota (*Bifidobacterium* and *Lactobacillus* genera). The most common prebiotics are non-digestible oligosaccharides (NDOs), such as fructooligosaccharides and inulin. Several studies investigated the prebiotic effects of other NDOs, such as xylooligosaccharide, galactooligosaccharide, and isomaltoligosaccharide (Roberfroid, 1997; Van Loo et al., 1999; Rabiou et al., 2001; Rycroft et al., 2001).

Generally, the intestinal microbial flora has a major effect on gastrointestinal (GI) function and in turn on human health. Probiotics, which usually belong to the *Bifidobacterium* and *Lactobacillus* genera, are live microbial ingredients that benefit the host health via colonisation of the lower intestine (Kolida et al., 2002). *Bifidobacterium* species exhibit several health-promoting effects on the GI tract. For instance, when regularly consumed as fermented food products, *Bifidobacterium* species serve as antimicrobial agents against various enteric pathogens, decrease serum cholesterol levels, reduce the incidence of colon cancer, and prevent gut disorders (Servin, 2004; Ljungh and Wadström, 2006; Sela et al., 2008; Cronin et al., 2011; Malaguamera et al., 2012).

Recently, oligosaccharides obtained from chitosan [chitooligosaccharides (COSs)] and low-molecular-weight chitosan have been claimed to also exhibit prebiotic effects (Macchi, 1996; Lee et al., 2002; Pan et al., 2009). COS are partially hydrolysed product of chitosan, a biopolymer composed of β -(1-4)-linked N-acetyl-D-glucosamine and deacetylated glucosamine units (Lin et al., 2007). Interest has been recently focused on their solubility in acid-free aqueous media, as opposed to high-molecular-weight chitosan. Furthermore, such oligomers have more potential than chitosan as a nutraceutical additive because COSs are easily absorbed through the intestine, thus quickly entering the blood flow and eliciting systemic biological effects (fermented by the intestinal microflora) in the organism (Chae et al., 2005; Fernandes et al., 2008). Lee et al. (2002) reported that COS with DP 2 - 8 have a bifidogenic effect at concentrations between 0.1% and 0.5% whereas COS have a growth stimulatory effect on *L. casei* and *L. brevis* at a concentration of 0.1%. Since these oligosaccharides are fully deacetylated, they are not digestible by the intestinal enzymes increasing the applicability of COS as prebiotics. Recently, Pan et al. (2009) have investigated the ability of 90% deacetylated COS ranged from 3 to 6 to act as prebiotics using the mouse model system. The study highlighted the concentrations of bifidobacteria and lactobacilli were increased while the concentrations of unfavourable enterococcus and *Enterobacteriaceae* in the caecum of mice were reduced when treated with COS for 14 days. As aforementioned, prebiotics should elicit the capability to stimulate the growth of probiotic bacteria. The main objective of this study was to evaluate the effect of COS upon the growth of *Bifidobacterium* sp. We also examined the pH changes by *Bifidobacterium* strains.

Materials and Methods

Materials and Organism

Chitosan was purchased from Eastern Global Sdn. Bhd. (Parit Buntar, Perak, Malaysia). Fruitafit® Inulin IQ, a commercial prebiotic, was obtained from Sensus (Roosendaal, Netherlands). de Man Rogosa Sharp (MRS) agar and broth from Merck, Germany was used throughout the study. The strains *Bifidobacterium breve* ATCC 15700 and *Bifidobacterium*

bifidum ATCC 11863 were purchased from Microbiologics, Inc (St. Cloud, Minnesota, USA). These strains were selected because they have potential probiotic properties.

Preparation of Samples by Enzymatic Hydrolysis

A total of 3% (w/v) chitosan was completely dissolved in 0.2 M acetate buffer (pH 4.5). The solution in the reaction vessel was placed in an incubator shaker (Ecotron: Infors-HT; Bottmingen, Switzerland) at 49.8 °C, and 25% (v/w) Celluclast® was added to initiate the reaction. After 24 h, the mixture was taken out and boiled for 10 min to stop the enzyme reaction. UF membranes with 10 kDa molecular weight cut-off were used to remove the enzyme. The filtrates were concentrated to about 1/20 with a rotary evaporator under reduced pressure. The precipitates were thoroughly washed with ethanol and then dried using a freeze dryer (FreeZone 4.5, Labconco; Kansas City, MO, USA).

Chromatography and Mass Spectrometry Condition

The components of COS were analysed by Synapt G2 HDMS Quadrupole Time-of-flight (QTOF/MS) Mass Spectrometer (Waters, Milford Massachusetts, USA).

Culture and Culture Conditions

The *Bifidobacterium* stock cultures were maintained at -80 °C in 0.05% L-cysteine MRS Broth (Merck, Germany) with 15% (wt/vol) glycerol until further analysis. For the prebiotic activity assay, 1 mL frozen glycerol stock cultures (1×10^5 CFU/mL) of *B. breve* ATCC 15700 and *B. bifidum* ATCC 11863 were transferred into 9 mL MRS broth and then incubated anaerobically for 48 and 96 h, respectively. Approximately 1 mL of overnight cultures (*B. breve* ATCC 15700 and *B. bifidum* ATCC 11863) was inoculated into 9 mL of MRS broth with the following components: 5% lactose, 5% inulin, 5% COS and 5% chitosan. The media were then incubated at 37 °C for 48 h under anaerobic conditions using GasPak anaerocult jar with Anaerocult® A sachet. Colony counts were done with 12 h interval until 48 h (Dubey and Mistry, 1996).

Bacterial Enumeration

Bacterial enumeration was performed in duplicates on MRS agar (Merck, Germany) plates at 0, 12, 24, 36 and 48 h. The inoculated MRS agar plates were incubated at 37 °C for 48 h in an anaerobic condition generated using Anaerocult® A sachet (Fernandes et al., 2012). Specific growth rate (k), defined based on doubling rate, is used as a way of measuring how fast cells are dividing. This parameter was calculated as follows (Kamaly, 1997);

$$k(h^{-1}) = 2.303(\log_{10} X_2 - \log_{10} X_1)/(t_2 - t_1)$$

where X_2 and X_1 are the cell or colony counts at times t_2 and t_1 , respectively.

Determination of pH

The pH of the sample at every 12 h interval for 48 h was measured with a pH metre PB-11 (Sartorius, Goettingen, Germany).

Statistical Analysis

Differences between specific growth rates were verified by ANOVA using MINITAB14 software for the pure culture fermentation of *Bifidobacterium* strains. Differences were considered to be statistically significant at $p < 0.05$.

Results

Mass Spectrometry Analysis of Hydrolysis Product

Fig. 1 shows the QTOF/MS, which revealed that the products were composed mainly of COS, especially DP 3 to 6 (Izume et al., 1992; Aiba, 1994a; Aiba, 1994b).

Growth of *Bifidobacterium* Species Cultivated with Prebiotic Carbohydrates

The cell growth of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 cultivated in MRS broth supplemented with 5% COS, 5% inulin, 5% lactose and 5% chitosan for 48 h at 37 °C was measured (Fig. 2). COS was found to have a similar growth effect as lactose and inulin on *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700. Except for chitosan, the amount of cell growth for all test samples significantly increased within 36 h of incubation. After 36 h of incubation, the cell growth remained relatively stable until 48 h of cultivation. Decrease of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 survivability in chitosan medium was observed after inoculation.

Effect of COS on the Specific Growth Rate of *Bifidobacterium* spp.

The specific growth rates of the *Bifidobacterium* spp. cultured in MRS supplemented with lactose, inulin and COS are summarised in Table 1. The specific growth rates of *B. bifidum* and *B. breve* in lactose, COS and inulin at 12 h were 0.32 and 0.30 h⁻¹, 0.31 and 0.24 h⁻¹ and 0.35 and 0.20 h⁻¹, respectively.

Changes in pH During Fermentation of COS

The changes in pH of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 and cultivated in MRS supplemented with lactose and prebiotic carbohydrates are presented in Table 2. The acid development of lactose, inulin, and COS fermented with *B. bifidum* ATCC 11863 after 12 h increased rapidly. The changes in pH (range: 5.16–5.19 to 4.64–4.67) of *B. bifidum* ATCC 11863 cultivated with lactose and inulin were greater than that with COS.

The pH changes was species-dependent with *B. breve* ATCC 15700 showed a greater reduction in pH than *B. bifidum* ATCC 11863 (Table 2). The pH changes for *B. bifidum* ATCC 11863 in the medium with substrate lactose, COS and inulin showed similar decreasing pattern from initial pH (5.16-5.22) to pH (4.64-4.74). pH changes for *B. breve* ATCC 15700 in the medium with substrate lactose, COS and inulin decreased from initial pH (5.15-5.18) to pH (4.29-4.55). For chitosan, pH changes from 6.40 to 6.33 and from 6.34 to 6.25 were observed for *B. breve* ATCC 15700 and *B. bifidum* ATCC 11863, respectively. These values remained relatively stable over 36 h of incubation.

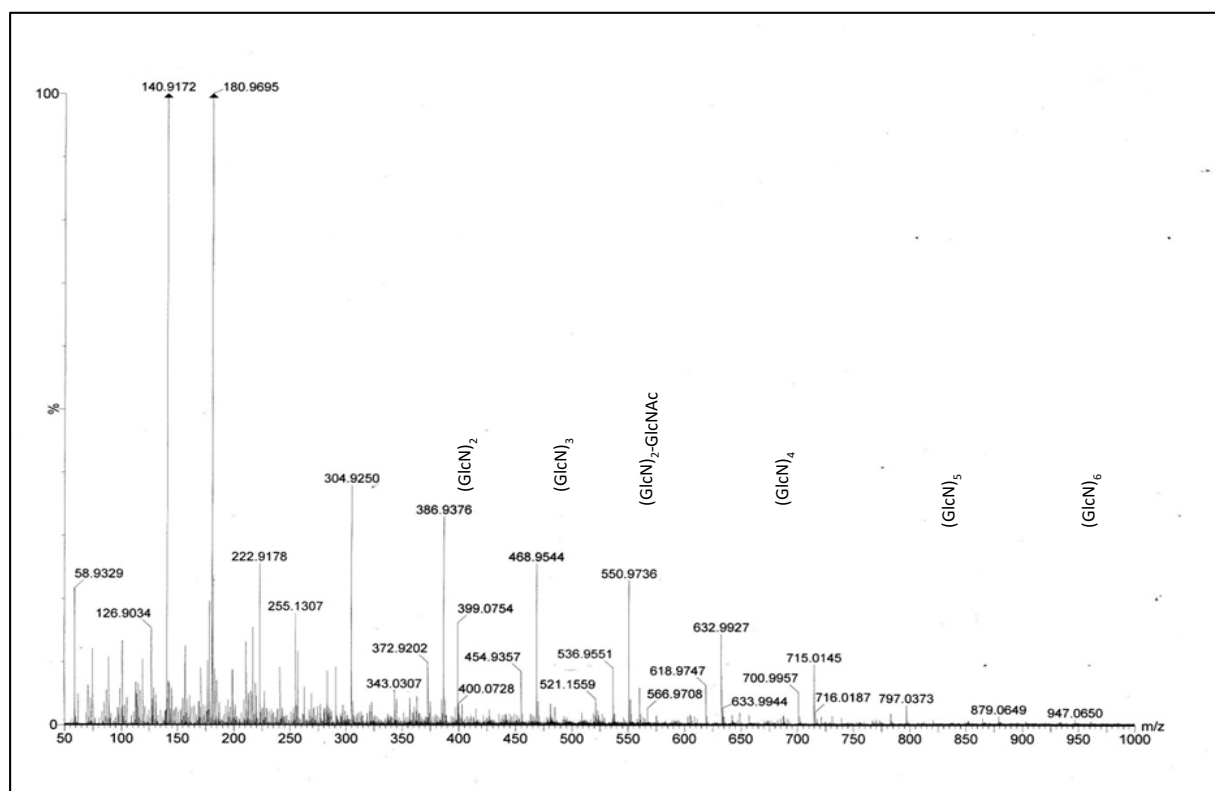


Figure 1. QTOF/MS of products from hydrolysis of chitosan by cellulase

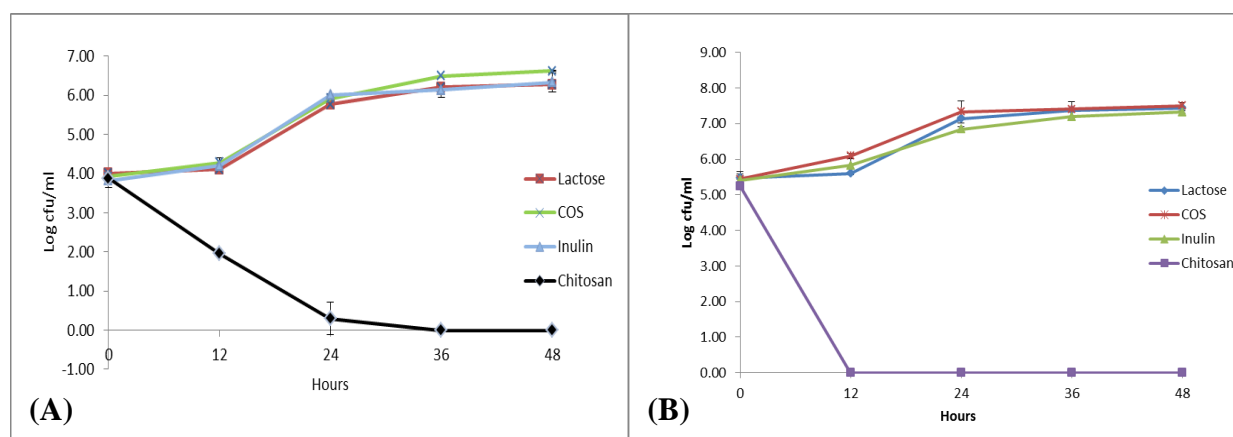


Figure 2. Effect of chitosan oligosaccharide (COS) on the growth of (A) *Bifidobacterium bifidum* ATCC 11863 (B) *Bifidobacterium breve* ATCC 15700 at different cultivation times. Results are given as means \pm SD of triplicate samples.

Discussion

QTOF-MS Analysis of COS

COS was prepared by enzymatic hydrolysis and QTOF/MS showed that the oligomer (2 dp to 6 dp) was completely deacetylated (Figure 1). Cellulase can hydrolyse chitosan to yield (GlcN)₃, (GlcN)₄, (GlcN)₅, and (GlcN)₆. This observation indicates that cellulase can split the β -1,4-glycosidic linkages of GlcN–GlcN. Cellulase can also produce (GlcN)₂–GlcNAc, (GlcN)₂–(GlcNAc)₂, (GlcN)₃–GlcNAc, (GlcN)₃–(GlcNAc)₂, (GlcN)₄–GlcNAc, and (GlcN)₄–(GlcNAc)₂.

(GlcNAc)₂. These results suggest that cellulase can selectively cleave GlcNAc–GlcN linkage, and that the hydrolysate is a mixture of heterooligomers, each of which carries a GlcNAc residue at the reducing end (Kittur et al., 2003).

COS Effect upon the Growth of *Bifidobacterium* sp

It is now well established that the colonic micro flora has a profound influence on health (Steer *et al.*, 2000). Consequently, there is currently a great deal of interest in the use of prebiotic oligosaccharides as functional food ingredients to manipulate the composition of colonic microflora in order to improve health (Gibson and Roberfroid, 1995; Gibson and Fuller, 2000; Roberfroid, 2000; Hammes and Hertel, 2002; Losada and Ollerios, 2002). Prebiotic oligosaccharides stimulate the growth and colonisation of probiotic bacteria, that is, non-pathogenic organisms that when ingested are beneficial to health (Rastall and Maitin, 2002). Therefore, the effectiveness of a prebiotic depends on its ability to be selectively fermented by and to support the growth of target organisms. The growth (calculated as log CFU/mL) of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 cultivated with COS showed a similar growth effects with lactose and inulin. Except for chitosan, the amount of cell growth for all test samples significantly increased within 36 h of incubation. After 0 h of incubation, no growth of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 in chitosan medium was observed. The population decreased between 12 and 36 h of incubation. This finding may be attributed to the lack of enzymes that are capable of digesting and utilising large chitosan molecules (Vernazza et al., 2005). This finding showed that the similar growth on COS, inulin and lactose, thus we agree with Huebner et al. (2007) who concluded that for a given sugar to have a prebiotic activity, it should be metabolised by a test strain as well as lactose. Lee *et al.* (2002) reported COS has a bifidogenic effect at concentrations between 0.1% and 0.5% although intestinal enzymes and lysozymes fully digested deacetylated COS. Moreover, COS at a concentration of 0.1% has a growth stimulatory effect on *Lactobacillus casei* and *Lactobacillus brevis*.

Table 1. Specific growth rate of *Bifidobacterium* species in 5% lactose, 5% chitosan oligosaccharide (COS) and 5% inulin

Culture ^b	Medium	Specific growth rate, k (h ⁻¹) ^a		
		0 h	12 h	24 h
<i>B. bifidum</i> ATCC 11863	Lactose	0.02 ^{cA}	0.32 ^B	0.08 ^{dA}
	COS	0.07 ^{dA}	0.31 ^B	0.11 ^{dA}
	Inulin	0.07 ^{dA}	0.35 ^B	0.03 ^{cA}
<i>B. breve</i> ATCC 15700	Lactose	0.02 ^{cA}	0.30 ^{cB}	0.04 ^{cA}
	COS	0.13 ^{eB}	0.24 ^{dC}	0.02 ^{cA}
	Inulin	0.08 ^{dA}	0.20 ^{dB}	0.07 ^{dA}

^a Values represent averages of triplicates experiments.

^b Initial cell population of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 was approximately 1 × 10⁵ CFU/mL.

^{c-e} Data in the same column with different lowercase letter is different significantly (p<0.05).

^{A-C} Data in the same row with different uppercase letter is different significantly (p<0.05).

Table 2. pH changes of *Bifidobacterium bifidum* ATCC 11863 and *Bifidobacterium breve* ATCC 15700 incubated for 48 h in MRS supplemented with different substrates.

Medium	pH ^a									
	<i>Bifidobacterium bifidum</i> ATCC 11863					<i>Bifidobacterium breve</i> ATCC 15700				
	0 h	12 h	24 h	36 h	48 h	0 h	12 h	24 h	36 h	48 h
Lactose	5.16 ± 0.03 ^{cA}	5.12 ± 0.09 ^{cA}	5.03 ± 0.09 ^{cA}	4.83 ± 0.20 ^{cB}	4.64 ± 0.18 ^{cB}	5.15 ± 0.06 ^{cA}	5.13 ± 0.05 ^{cA}	4.70 ± 0.07 ^{cB}	4.43 ± 0.08 ^{cC}	4.29 ± 0.06 ^{cC}
COS	5.22 ± 0.02 ^{cA}	5.21 ± 0.06 ^{cA}	5.21 ± 0.1 ^{dA}	5.02 ± 0.22 ^{cB}	4.74 ± 0.13 ^{cC}	5.18 ± 0.07 ^{cA}	5.20 ± 0.06 ^{cA}	4.98 ± 0.01 ^{dB}	4.69 ± 0.07 ^{dC}	4.55 ± 0.04 ^{dC}
Inulin	5.19 ± 0.01 ^{cA}	5.21 ± 0.00 ^{cA}	5.09 ± 0.13 ^{cA}	4.84 ± 0.18 ^{cB}	4.67 ± 0.13 ^{cB}	5.15 ± 0.06 ^{cA}	5.14 ± 0.07 ^{cA}	4.72 ± 0.05 ^{cB}	4.52 ± 0.09 ^{cC}	4.39 ± 0.03 ^{cC}
Chitosan	6.40 ± 0.01 ^{dA}	6.43 ± 0.01 ^{dA}	6.41 ± 0.08 ^{eA}	6.33 ± 0.20 ^{dA}	6.33 ± 0.14 ^{dA}	6.34 ± 0.04 ^{dA}	6.32 ± 0.08 ^{dA}	6.35 ± 0.11 ^{eAB}	6.25 ± 0.07 ^{eB}	6.25 ± 0.05 ^{eB}

^a Values represent averages of triplicate experiments

^b Initial cell population of *B. bifidum* ATCC 11863 *B. breve* ATCC 15700 was approximately 1 x 10⁵ CFU/mL

^{c-d} Data in the same column with different lowercase letter is different significantly (p<0.05)

^{A-C} Data in the same row with different uppercase letter is different significantly (p<0.05)

When comparing the two strains of *Bifidobacterium* species, the cell growth of *B. breve* ATCC 15700 was higher than that of *B. bifidum* ATCC 11863 under the same conditions (48 h, 37 °C) and inoculum amount (10^5 CFU/mL). The slow growing strain *Bifidobacterium bifidum* var *pennsylvanicus* (ATCC 11863) was found to have a high N-acetyl-D-glucosamidase activity. This organism, isolated from the stools of breast-fed and bottle-fed infants, require N-acetyl-D-glucosamine-containing saccharides as substrates for cell wall synthesis (Poupard et al., 1973). The differences in growth rates among the species of *Bifidobacterium* might be also explained by their different levels of tolerance to aerobic conditions. Obligate anaerobic species might have grown poorly because of the insufficient oxidation-reduction potential of the medium. The growth of anaerobic organisms might be stimulated by the addition of L-cysteine to decrease the oxidation-reduction potential of the medium. The inoculum was grown in MRS supplemented with yeast extract and L-cysteine (Desjardins et al., 1990).

From the findings, at 12 h the growth rate of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 in lactose was higher than that in COS and inulin, with a significant difference ($p < 0.05$) at 24 h of fermentation (Table 1). In other incubation periods, there were significant differences ($P < 0.05$) in growth rate between the strains cultured in COS, lactose and inulin although COS tended to show the highest specific growth rate for *B. bifidum* ATCC 11863. The growth rate of the strains in COS and inulin for different times differ significantly ($p < 0.05$). When comparing the growth rate for different times (horizontal comparison), the increase in the growth rate of *B. bifidum* ATCC 11863 cultured in inulin was significantly higher ($p < 0.05$) than that in lactose and COS. The growth rates of the *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 in COS and lactose differ significantly ($p < 0.05$) after 0 h to 24 h of fermentation, the trend of the growth is the same as that in inulin. These results indicate that the growth rate of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 in COS is similar to that in inulin and lactose. Therefore, COS could be used as a sole carbon source for the growth of the human gut microflora.

The decrease in pH was dependent on the species. *B. breve* ATCC 15700 showed a greater decrease in pH than *B. bifidum* ATCC 11863. This finding may be attributed to the fact that *Bifidobacterium* species produce 3 mol acetic acid and 2 mol lactic acid for each two mol glucose (Scardovi, 1986). The production of these two organic acids and low pH values in the colon are among the probiotic factors contributing to the well-being of the host in which they will suppress the growth of unwanted microorganisms in the gut (Crittenden, 1999). Scardovi (1986) stated that *Bifidobacterium* species have a narrow optimum pH range (6.5 to 7) for growth and that no growth occurs at pH < 5 or > 8 . Desjardins et al. (1990) revealed that most *Bifidobacterium* strains demonstrate a relationship between growth rate and acid production. This finding is consistent with the results of the present study.

Conclusion

This study investigated the effects of chitosan and its oligosaccharides on the growth of *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700. The amount of cell growth for all medium samples, except for the chitosan medium, significantly increased within 48 h of incubation. COS was found to have a similar effect on *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 with inulin and lactose. The pH changes of COS, inulin and lactose fermented with *B. bifidum* ATCC 11863 and *B. breve* ATCC 15700 after 12 h increased rapidly. The specific growth rate of *bifidobacterium* species was higher at 12 h and *B. bifidum* ATCC 11863 showed the highest specific growth rate. Observation of both strains demonstrated that a relationship exists between growth rate and acid production.

The study has shown prospects for COS as a functional food, a prebiotic in promoting the growth of bifidobacteria. Thus further investigation of the full range of chitosan molecules reaching the gut should thus be undertaken to verify the safety of these molecules.

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